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Abstract: Non-typhoid Salmonella strains containing virulence plasmids are highly associated with bacteremia and disseminated infection in humans. These plasmids are found in Salmonella serovars adapted to domestic animals, such as Salmonella dublin and Salmonella choleraesuis, as well as in the widely distributed pathogens Salmonella typhimurium and Salmonella enteritidis. Although virulence plasmids differ between serovars, all contain a highly conserved 8-kb region containing the spv locus that encodes the spvR regulatory gene and four structural spvABCD genes. Studies in mice suggest that the spv genes enhance the ability of Salmonella strains to grow within cells of the reticuloendothelial system. The spv genes are not expressed during exponential growth in vitro but are rapidly induced following entry of Salmonella strains into mammalian cells, including macrophages. Transcription of the spv genes is controlled by the stationary-phase (T factor RpoS, and mutations in RpoS abolish virulence. These studies suggest that the ability of Salmonella strains to respond to starvation stress in the host tissues is an essential component of virulence

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Biology and Clinical Significance of Virulence Plasmids in *Salmonella* Serovars

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Non-typhoid *Salmonella* strains containing virulence plasmids are highly associated with bacteremia and disseminated infection in humans. These plasmids are found in *Salmonella* serovars adapted to domestic animals, such as *Salmonella dublin* and *Salmonella choleraesuis*, as well as in the widely distributed pathogens *Salmonella typhimurium* and *Salmonella enteritidis*. Although virulence plasmids differ between serovars, all contain a highly conserved 8-kb region containing the *spv* locus that encodes the *spvR* regulatory gene and four structural *spvABCD* genes. Studies in mice suggest that the *spv* genes enhance the ability of *Salmonella* strains to grow within cells of the reticuloendothelial system. The *spv* genes are not expressed during exponential growth in vitro but are rapidly induced following entry of *Salmonella* strains into mammalian cells, including macrophages. Transcription of the *spv* genes is controlled by the stationary-phase σ factor RpoS, and mutations in RpoS abolish virulence. These studies suggest that the ability of *Salmonella* strains to respond to starvation stress in the host tissues is an essential component of virulence.

Organisms of the genus *Salmonella* cause three distinct clinical syndromes: (1) gastroenteritis, (2) enteric (typhoid) fever, and (3) septicemia, with frequent metastatic foci of infection. The most common manifestation of salmonella infection is self-limited enteritis, and dissemination of bacteria outside of the gastrointestinal tract is unusual in the healthy host. In contrast, systemic disease is the hallmark of enteric fever, with bacteremia and infection of lymphoid tissue and the reticuloendothelial system. Clinical gastroenteritis is not prominent, but late complications of Peyer's patch infection, which involve perforation and hemorrhage, are particularly distinctive features of enteric fever and are uncommon in other salmonella infections. Disseminated disease is also seen in the third form of salmonellosis, but in this syndrome, severe sepsis may dominate the clinical picture or patients may present with localized foci of infection; gastrointestinal symptoms may be mild or absent, and the bowel complications seen in enteric fever are not present [1].

Although a very large number of distinct *Salmonella* strains have been identified by a combination of serological, biological, and genetic techniques, a limited number of strains have been shown to be closely associated with either the enteric fever or septicemia syndromes. Only *Salmonella typhi* and, occasionally, *Salmonella paratyphi* serovars have been shown to cause typhoid fever. These strains are host adapted to humans and do not cause disease in animals. Both *Salmonella choleraesuis* and *Salmonella dublin* are strongly associated with the septicemic/metastatic form of salmonellosis, while these organisms are uncommon causes of gastroenteritis in humans [1, 2]. In contrast, >2,000 different serovars have been identified among isolates from patients with gastroenteritis, and a few of these, notably *Salmonella typhimurium* and *Salmonella enteritidis*, are also prominent isolates in cases of salmonella bacteremia [2].

These observations indicate that *Salmonella* strains differ in their ability to produce particular disease syndromes. The molecular genetic basis for the different virulence traits among these strains remains poorly understood. However, recent evidence indicates that a common plasmid-encoded genetic locus is a major determinant of virulence in non-typhoid *Salmonella* serovars associated with systemic disease [3, 4]. Virulence plasmids in *Salmonella* strains were identified by several early studies in which large "cryptic" plasmids were cured, and the plasmid-free derivatives were found to be less virulent in mice. Subsequent studies in which virulence was restored by reintroduction of the plasmids provided definitive evidence for plasmid-encoded virulence genes [3, 4].

Distribution of Virulence Plasmids in *Salmonella* Serovars

Virulence plasmids are found in only a small number of *Salmonella* serovars, and these can be divided into two groups based on their epidemiology. One group consists of serovars that are host adapted to domestic animals: *S. dublin* (cattle),

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S. choleraesuis (pigs), *Salmonella gallinarum-pullorum* (fowl), and *Salmonella abortusovis* (sheep) [5, 6]. The host adaptation is reflected by the observations that the particular serovar is frequently associated with disseminated disease in its host species but is usually not found in other animal species. *S. dublin* and *S. choleraesuis* are also associated with systemic disease in humans, while *S. gallinarum-pullorum* and *S. abortusovis* are not human pathogens. It is significant that virulence plasmids have not been found in *S. typhi,* despite its properties of human adaptation and propensity to cause systemic illness [5]. The second group of *Salmonella* strains carrying virulence plasmids are the broad host-range serovars *S. typhimurium* and *S. enteritidis* [5]. These organisms are isolated from a variety of hosts and are prominent causes of both the gastroenteritis and septicemia syndromes in humans.

Virulence plasmids range in size from 50 kb to 100 kb, and considerable differences exist between plasmids from individual serovars. However, plasmids isolated from strains of a given serovar are remarkably similar. These observations suggest that the plasmids are specific to the particular serovar and that little if any plasmid exchange occurs between serovars. These findings are consistent with the apparent absence of complete self-transmissible conjugation systems on the virulence plasmids [7]. Heteroduplex analysis indicates that the larger *S. typhimurium* plasmid (100 kb) contains a region hybridizing to virtually all of the *S. enteritidis* plasmid (60 kb) and almost all of the *S. choleraesuis* plasmid (50 kb). In contrast, large regions of the *S. dublin* plasmid (80 kb) appear unrelated to any of the sequences of the other virulence plasmids tested [8]. Despite these differences, the plasmid from *S. enteritidis* was found to restore mouse virulence to a plasmid-cured *S. dublin* strain [9]. In a similar vein, the plasmid from *S. gallinarum-pullorum* is able to substitute for the *S. typhimurium* plasmid in mouse virulence, despite the fact that *S. gallinarum-pullorum* strains are not virulent in mice [10]. The results indicate that virulence plasmids are not responsible for the host-adaptation phenotype, but rather encode a common virulence function that is interchangeable among the host serovars. Subsequent studies have identified a highly conserved region of ~8 kb, designated *spv* for *Salmonella* plasmid virulence, present on all the plasmids and responsible for the virulence phenotype in mice. The *spv* locus consists of the *spvR* regulatory gene and four structural genes, *spvABCD.*

Contribution of the *spv* Locus to the Virulence of *Salmonella* Serovars

Most of the work on the virulence plasmid phenotype has been done with inbred mice homozygous for the Ity^S locus. These mice are much more susceptible to *Salmonella* strains than are animals carrying the Ity^R allele. In Ity^S mice, *S. typhimurium, S. dublin, S. enteritidis,* and *S. choleraesius* strains cured of their respective plasmids are 10^1- to 10^6-fold less virulent than their isogenic wild-type parent strains [3, 4]. This loss of virulence is generally seen whether the inoculum is given intraperitoneally or orally, although certain *S. typhimurium* strains may show a plasmid effect only with oral infection [11]. The plasmid virulence phenotype is also expressed in Ity^R mice. Detailed studies on the pathogenesis and histopathology of oral salmonella infection in mice show that the plasmid is not required for invasion of the bacteria into intestinal epithelial cells and Peyer's patches, nor for spread to mesenteric nodes and the reticuloendothelial system (RES) of the liver and spleen. Instead, the plasmid-containing strains outgrow plasmid-free bacteria in the RES, leading to an overwhelming infection characterized by prominent microabscess formation and large numbers of organisms in the liver and spleen prior to death at 6–8 days after inoculation [12]. The plasmid-free strain of *S. dublin* was found to invade the bowel wall and ileal Peyer's patches with an efficiency equal to that of the wild-type parent strain. However, plasmid-free bacteria in the mesenteric lymph nodes and the spleen increased much more slowly than wild-type, remaining 10^2- to 10^3-fold lower in number at 7 days after infection. After 10 days, the number of plasmid-free bacteria decreased and the liver histology showed numerous small granulomas. These results indicate that the slower growth of plasmid-free strains allows the host to mount an effective immune response. Polymorphonuclear leukocytes do not appear to be important in controlling the initial growth of plasmid-free strains, since the numbers of bacteria recovered from neutropenic mice over the first 7 days of infection were the same as those for control mice [12].

Several lines of evidence suggest that the virulence plasmid enhances bacterial growth within the intracellular environment of host cells. In the murine model, *Salmonella* strains rapidly enter cells following inoculation [13]. Gentamicin treatment of mice infected with plasmid-containing *S. dublin* does not alter the lethal course of infection, owing to the inability of the antibiotic to enter host cells. However, mice are cured of salmonella infection by the administration of gentamicin incorporated into liposomes, which enables delivery of the drug to the intracellular environment [14]. Results of studies in which a temperature-sensitive marker plasmid is used in *S. typhimurium* suggest that the virulence plasmid accelerates intracellular growth of bacteria in the liver and spleen [15], and recent evidence indicates that the vast majority of viable plasmid-containing bacteria in the spleen are found in the macrophages within 3 days after infection (N. A. Buchmeier, unpublished observations). Plasmid-containing strains do not appear to cause any general immunosuppression, since the growth of plasmid-free strains is not enhanced by mixed infection [16]. Results of these in vivo studies suggest that the virulence plasmid increases bacterial growth within macrophages of the RES, although growth within other host cells may occur as well.

Only limited studies of the virulence plasmid phenotype have been done in humans and domestic animal hosts. The virulence plasmid is required for *S. gallinarum-pullorum* to produce severe systemic disease in fowl [17]. The *S. choleraesuis* plasmid

also appears to enhance disease in pigs [18]. Since only a portion of *S. typhimurium* strains carry a virulence plasmid, it is possible to use molecular epidemiology to determine whether plasmid-containing strains are more closely associated with systemic disease than with gastroenteritis. By using this approach, we have found that 76% of strains isolated from blood cultures but only 42% of unrelated fecal isolates carried a virulence plasmid; these findings provide evidence that the virulence plasmid plays a significant role in human disease [19]. Similar results have also been reported in studies of domestic livestock [8]. These findings confirm the general significance of virulence plasmids in systemic disease due to nontyphoid *Salmonella* serovars.

The absence of virulence plasmid sequences in *S. typhi* and the *S. paratyphi* serovars associated with enteric fever suggests that the pathogenesis of typhoid is quite different from that of disseminated, non-typhoid salmonellosis. Another difference is the presence of the Vi capsule in *S. typhi* and its absence in most virulence plasmid–containing isolates. *S. typhi* is not pathogenic for mice, and introduction of a virulence plasmid does not restore virulence in murine infections [5]. This result indicates that crucial differences in chromosomal genes exist between *S. typhi* and the mouse virulent serovars. Chromosomal loci required for mouse virulence are also missing from a number of non-typhoid serovars. Transfer of a virulence plasmid to the *Salmonella* serovars *S. derby, S. havana, S. minnesota, S. ohio,* and *S. saintpaul* failed to confer mouse virulence on these strains, while *S. heidelberg* and *S. newport* were rendered virulent [5]. These studies indicate that both the virulence plasmid and the chromosome are key determinants of mouse virulence. Since *S. typhi* differs in both of these elements, salmonellosis in mice has severe limitations as a model for typhoid fever and more likely parallels the septicemia syndrome.

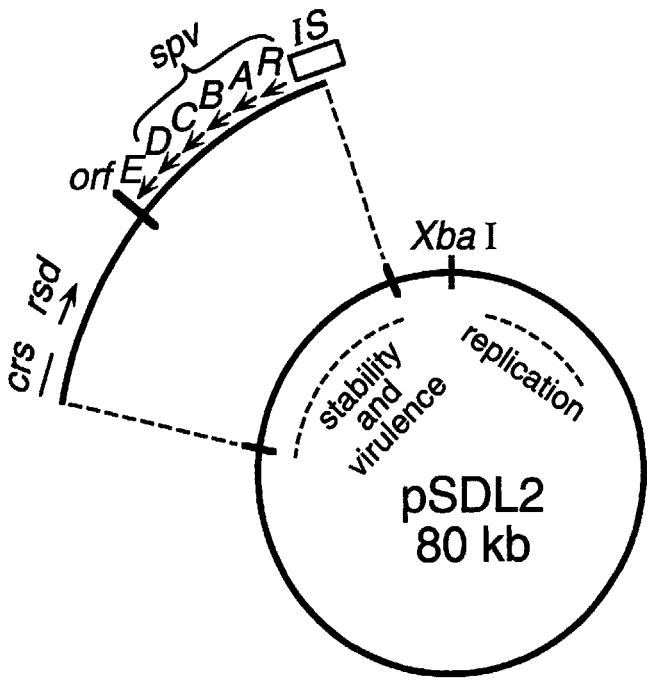

Figure 1. Map of known genetic loci on the *Salmonella dublin* virulence plasmid pSDL2. A region encoding autonomous replication functions is located clockwise from the single *Xba* I site. The essential virulence locus consists of the five *spvRABCD* genes, bordered by a IS630-like element upstream and *orfE* downstream. A region affecting plasmid stability constitutes a multimer resolution system, composed of a gene for resolvase (*rsd*) and a crossover site for specific recombination (*crs*), leading to the conversion of plasmid multimers to monomers.

Molecular Analysis of the *S. dublin* Plasmid pSDL2

In early studies in which insertion mutations and deletions were used, a single virulence region on the *S. dublin* plasmid was identified, as well as a fragment capable of autonomous replication (figure 1) [9]. Subsequent experiments showed that an 8.2-kb segment from the virulence region cloned on an IncP plasmid replicon could restore virulence to a plasmid-cured *S. dublin* strain. Complete sequence analysis of this fragment revealed six open reading frames, currently designated *spvRABCD* and *orfE* (figure 1) [20]. Comparison with *spv* sequences from *S. typhimurium* and *S. choleraesuis* show <0.5% divergence at the nucleotide level [3]. An insertion sequence–like element related to IS630 is located upstream from *spvR* [21]. Early work identified a region downstream from *orfE* required for plasmid stability [9]. This region was found to contain a resolvase (*rsd*) closely related to the D protein of miniF and a *cis*-acting resolution site, designated *crs* [22]. The *crs* element contains eight direct, incomplete 17-bp repeats and a region of indirect repeats homologous to *oriV1* of F. The *rsd/crs* system is capable of resolving multimers generated in *recA*$^+$ hosts and of stabilizing heterologous replicons in *Salmonella* strains. A resolvase function active on *crs* was found in other *Salmonella* serovars harboring a virulence plasmid, suggesting that this system is a general mechanism for virulence plasmid stabilization.

Deletion and insertion mutagenesis has been used to define the plasmid genes essential for virulence in *S. dublin.* Tn*5-oriT* insertions in *orfE* retain virulence, but deletion of *spvD* partially attenuates virulence [20]. Tn*5-oriT* inserts in *spvR, spvB,* and *spvC* abolish virulence [20]. Due to the polar nature of Tn*5* inserts, non-polar mutations using an *Xba* I linker oligonucleotide were also constructed [23]. Analysis of these plasmids showed that *spvA* is not essential for virulence in mice. Mutations throughout *spvB* are nonvirulent, including stop-codon inserts close to the COOH-terminus. Mutations in *spvC* and *spvD* are partially virulent but the plasmids are unstable in vivo. Insertions in *spvR* are avirulent and abolish production of the SpvABCD proteins, consistent with the essential regulatory role of *spvR* (see below). Taken together, the results indicate that *spvR* and *spvB* are essential virulence genes in mice and that *spvC* and *spvD* have accessory roles and are probably needed for full virulence, while *spvA* is not essential. In

S. typhimurium, spvC and *spvD* have significant roles in mouse virulence [24, 25].

The *spv* genes are transcribed from two promoter regions, one located upstream from *spvR* and the second positioned between *spvR* and *spvA* [26]. Transcription of the *spvABCD* genes is initiated upstream from *spvA,* and messages for *spvA, spvAB, spvABC,* and *spvABCD* are found in decreasing abundance. LacZ translational fusions to the Spv proteins are also expressed with the same decreasing activity, SpvA being highest and SpvD lowest [26]. These findings suggest that transcripts initiate at the *spvA* promoter and can terminate within each of the long intergenic regions. However, a complex system of termination and mRNA processing cannot be excluded. The promoter for *spvR* has not been localized, but a monocistronic message is found, indicating that *spvR* transcription normally terminates before the *spvA* gene (M. Krause and D. G. Guiney, unpublished observations).

Regulation of *spv* Expression

The discovery that *spv* expression varies during the bacterial growth phase provided the key to understanding the mechanism of *spv* gene regulation shown in figure 2 [27]. When LacZ translational fusions were used, the expression of *spvABCD* was found to be very low in early log-phase cells and to increase rapidly in the post-exponential phase [26, 27]. This growth-phase regulation was confirmed by analysis of *spv* mRNA transcribed in wild-type *S. dublin* [26]. In the log phase, only small quantities of single-length *spvA* mRNA are made. As cells enter the stationary phase, progressively longer messages are found, including full-length *spvABCD* mRNA.

Sequence analysis of SpvR suggested that this protein belongs to the large family of LysR/MetR-like transcriptional activators [28]. These proteins act as single-component regulators of gene expression, and they contain a conserved helix-turn-helix motif in the NH_2-terminal region involved in DNA interaction [29]. Genetic studies confirmed that SpvR is absolutely required for *spvABCD* expression [27]. Knockout mutations in *spvR* abolish the synthesis of LacZ fusions with the structural Spv proteins, as well as production of the native Spv proteins in minicells [23, 26, 27]. SpvR binds to a region upstream from the spvA transcriptional start sites, and mutations in the helix-turn-helix motif abolish binding in vitro and activation of *spvA* transcription in vivo [30]. However, overproduction of SpvR from constitutive promoters does not abolish the growth-phase regulation of the *spvABCD* structural genes, indicating that the SpvR level is not the sole regulator of *spv* expression [26].

The pattern of *spv* growth-phase regulation strongly suggested the involvement of the alternative σ factor RpoS (KatF, σ^s). The activity of this σ factor is induced during post-exponential growth and regulates the stationary-phase expression of a large set of target genes involved in starvation survival and environmental stress responses [31]. The central role of RpoS in *spv* gene expression was demonstrated by constructing an *rpoS* knockout mutation in *S. typhimurium* [32]. Expression of *spvB* is severely decreased in the *rpoS* mutant. As expected, the *rpoS* mutant is markedly attenuated for virulence in mice. Recent evidence indicates that RpoS is required for optimal transcription at both the *spvR* and *spvA* promoters (figure 2) ([33] and D. G. Guiney, unpublished observations).

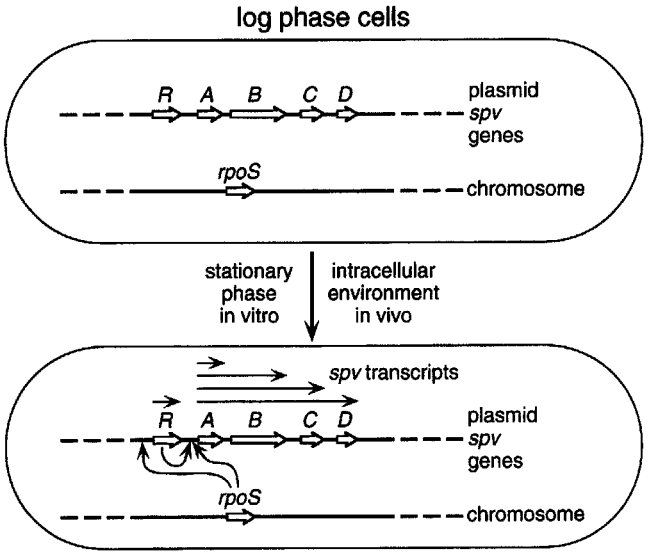

Figure 2. Mechanism of *spv* gene regulation on the virulence plasmids of *Salmonella* serovars. Bacterial cells growing in logarithmic phase in rich culture media do not express the *spv* genes (depicted in the upper cell). However, both post-exponential growth in vitro and the intracellular environment of host cells in vivo induce the *spv* genes (shown in the lower cell). Induction depends on both the chromosomal σ factor RpoS and the plasmid regulatory protein SpvR. RpoS activity increases in stationary phase and in response to nutrient starvation. RpoS appears to act at both the *spvR* and *spvA* promoters. SpvR is essential for transcription of the *spvABCD* genes by binding to the *spvA* promoter region. Transcription of the *spvABCD* structural genes is initiated at the *spvA* promoter, and multiple transcripts are found.

Induction of *spv* Gene Expression by the Host-Cell Environment

Since considerable indirect evidence suggests that the *spv* genes are active in the intracellular environment of the host, the expression of the *spv* genes was examined after uptake of bacteria by mammalian cells in tissue culture [34]. Fluorescent (and later luminescent) substrates of LacZ were used to develop a sensitive assay for the expression of *spv::lacZ* fusions by small numbers of bacteria inside eukaryotic cells. These studies have shown that the *spv* genes are rapidly induced by >100-fold on uptake by macrophages, epithelial cells, and hepatocytes ([34] and S. Libby, J. Fierer, and D. G. Guiney, unpublished data). Most of the induction occurs within the first hour following uptake and does not depend on acidification of the

intracellular vacuole containing the *Salmonella* strain. This regulation differs considerably from control of the chromosomal locus *pagC*; *pagC* is regulated by the PhoP/PhoQ two-component system and is induced in macrophages, but not epithelial cells, by a mechanism requiring phagosome acidification over a period of 3 hours [35].

These results indicate that *spv* induction occurs in response to a general property of the intracellular environment, perhaps reflecting a relative lack of nutrients available within the endocytic or phagocytic vacuole. Since the intracellular induction of the *spv* genes requires both SpvR and RpoS, the genetic mechanisms regulating post-exponential phase synthesis in vitro are the same as those controlling the *spv* operon after entry into host cells, and presumably throughout the infectious process (figure 2). The finding that virulence genes are regulated by RpoS suggests that *Salmonella* strains experience significant starvation stress during intracellular infection. Since the RpoS-mediated response increases bacterial resistance to a variety of adverse environmental factors, this regulation appears to be essential for the organism to survive and multiply in the host.

Plasmid-Cured *Salmonella* Strains as Live Vaccines

Since plasmid-cured *S. dublin* produces a self-limited systemic infection with granuloma formation, the ability of this strain to induce protective immunity was tested in mice [36]. Animals immunized by either oral or intraperitoneal inoculation were protected against lethal challenge with virulent *S. dublin* given by oral or intraperitoneal routes of infection. The fact that intraperitoneal vaccination could protect against a virulent oral challenge led to the demonstration that systemic as well as oral salmonella vaccination produces infection of Peyer's patches in the intestine. Mice immunized with *S. dublin* (group D) were protected against challenge with other group D *Salmonella* serovars and also against *S. typhimurium* (group B), which shares an O-antigen determinant with group D [36]. Similar results were reported with *S. enteritidis* (group D) [37]. However, *S. dublin* does not protect mice from *S. choleraesuis* (group C), which lacks any cross-reactive O-antigen epitopes [36]. It is likely that both lipopolysaccharide and non-lipopolysaccharide antigens are important in the protection induced by live, plasmid-cured strains. These immunization studies have important implications for vaccine development. The use of the plasmid-free strain as a vaccine shows that an immune response to the Spv proteins is not required for protection. Since plasmid-containing *Salmonella* serovars are major pathogens of domestic animals, plasmid-free derivatives could be combined in a multivalent vaccine for use in livestock.

Conclusions

The plasmid-encoded *spv* genes enhance the ability of certain non-typhoid *Salmonella* serovars to produce severe, extraintestinal disease. The *spv* locus consists of the *spvR* regulatory gene and the four structural *spvABCD* genes. Studies on *spv* gene regulation have established the importance of post-exponential or stationary-growth-phase control mechanisms on the expression of virulence genes in *Salmonella* strains. The alternative σ factor RpoS, together with SpvR, regulates expression of the *spv* operon and ensures rapid induction of the *spv* genes within the intracellular environment of the host. Evidence from pathogenesis studies in mice suggests that the *spv* genes enhance the growth of *Salmonella* species within cells of the RES.

In clinical practice, non-typhoid salmonella bacteremia and metastatic infection are associated with a variety of predisposing conditions that decrease natural or acquired immunity, including extremes of age, decreased gastric acidity, malignancy, immunosuppression, and AIDS. The *Salmonella* serovars causing systemic disease in these patients are predominantly strains that carry a virulence plasmid. The virulence-plasmid phenotype does not appear to interfere with clearance by acquired immune mechanisms. By extrapolation from the mouse model, the *spv* genes are likely to facilitate growth of the *Salmonella* strains within host cells at the sites of systemic infection, particularly in patients who are unable to mount an effective immune response. Salmonella infection in patients receiving immunosuppressive therapy or in patients with AIDS is particularly difficult to eradicate. The identification of the *spv* locus provides a molecular genetic explanation for the propensity of plasmid-containing serovars to cause serious infections in these patients.

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